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Proper Morphogenesis of the *Caenorhabditis elegans* Male Tail

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The *Caenorhabditis elegans* gene *lin-29* encodes a zinc-finger transcription factor that is required for hypodermal cell terminal differentiation and proper vulva morphogenesis. Here we demonstrate that *lin-29* is also required in males for productive mating. We show that *lin-29* males can perform the early mating behaviors including response to hermaphrodite contact and vulva location, but they do not perform the subsequent steps of vulva attachment via spicule insertion and sperm transfer. Consistent with this observation, we found that *lin-29* mutant spicules are on average 43% shorter than wild-type spicules while other male mating structures appear unaltered. In *lin-29* mutants, spicule development goes awry after the generation of spicule cells, when spicule morphogenesis occurs in wild-type males. We show that LIN-29 accumulates in many cells of the wild-type male tail, including those that form the spicules. We demonstrate, through analysis of genetic mosaics, that the formation of wild-type-length spicules requires *lin-29(+)* in the AB.p lineage, the lineage that gives rise to the spicules and other male copulatory structures. Our mosaic analysis also reveals a role for *lin-29(+)* in the P1 lineage, which mainly produces sex muscles, cells of the somatic gonad, and body wall muscles.

INTRODUCTION

Male tail development in *Caenorhabditis elegans* is a complex postembryonic process that requires the coordination of diverse cell types to produce a highly specialized structure required for mating. Approximately one-third of the 471 cells born postembryonically in the male are involved in the structure and function of the male mating apparatus (Sulston *et al.*, 1980). Additional cells born postembryonically comprise the somatic gonad, the development of which must coordinate with tail development to ensure sperm delivery (Sulston *et al.*, 1980).

The formation of a differentiated structure such as the male tail requires the integration of spatial, temporal, and sex-specific cues and involves the coordination of developmental events in different cell types and tissues. The mechanisms that guide these processes in male tail development are particularly amenable to genetic analysis because the self-propagating nature of the *C. elegans* hermaphrodite renders the male tail dispensable. Genetic screens for infertile males and males with morphologically defective tails have resulted in the identification of many genes required for male tail development (see Emmons and Sternberg, 1997, for review). Additional genes involved in the formation or function of the male tail have been identified because of their roles in other developmental processes. Here we report that *lin-29* is a member of this latter class of genes.

lin-29 was first described for its requirement in the execution of lateral hypodermal seam cell terminal differentiation (Ambros and Horvitz, 1984). The hypodermal seam cells are arranged linearly along each lateral midline of the body. During the three larval molts (L1-to-L2 molt through L3-to-L4) the seam cells divide in a stem-cell-like fashion and contribute to the synthesis of

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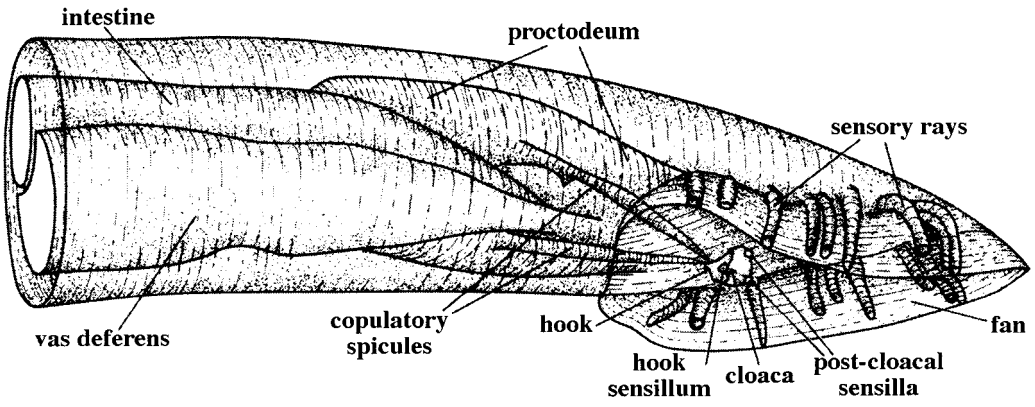

FIG. 1. Gross morphology of the male tail. Adapted from Sulston *et al.* (1980).

a larval-type cuticle. During the final molt (L4-to-adult) the seam cells terminally differentiate; they exit the cell cycle and fuse into bilateral syncytia that extend the length of the animal and secrete adult-type cuticle (Sulston and Horvitz, 1977; Singh and Sulston, 1978). In *lin-29* mutants, the lateral hypodermal seam cells remain in the proliferative larval state during the fourth molt: they fail to fuse and they synthesize larval-type cuticle rather than adult-type cuticle. In addition, the animals execute extra molting cycles not observed in the wild type (Ambros and Horvitz, 1984). *lin-29* encodes a zinc finger transcription factor, and its regulatory targets include cuticle collagen genes (Rougvie and Ambros, 1995). *lin-29* activity in the hypodermis is restricted to the final larval stage in wild-type animals by the action of the heterochronic genes, including *lin-4*, *lin-14*, and *lin-28* (Ambros, 1989; Bettinger *et al.*, 1996).

The proper timing of terminal differentiation in a small subset of lateral hypodermal cells is required for the final steps of vulva morphogenesis in hermaphrodites (Bettinger *et al.*, 1997), demonstrating the importance of temporal coordination of differentiation events in different cell types that contribute to a common structure or organ. Because many of the factors that control vulva development in hermaphrodites also play roles in male tail development (Chamberlin and Sternberg, 1994), we investigated whether *lin-29* controls any male-specific developmental processes. Here, we report that *lin-29* mutant males are mating-defective, and we characterize the cellular basis of this defect.

Male mating consists of a series of behaviors that culminates with insemination of the hermaphrodite. Male mating behaviors subsequent to hermaphrodite location have been analyzed and divided into a series of five steps (J. Sulston, personal communication; Liu and Sternberg, 1995): (1) response to hermaphrodite contact by backward movement with contact between the male's tail and the hermaphrodite's body, (2) turning when one end of the hermaphrodite's body is reached, (3) vulva location, (4) insertion of the copulatory spicules to attach the male tail to the vulva, and finally (5) sperm transfer. Productive execution of these steps requires proper development of the specialized male tail.

The main components of the male tail are the internal proctodeum and the external hook and sensory fan (Fig. 1; Sulston *et al.*, 1980; see Emmons and Sternberg, 1997, for review). The proctodeum houses the two retractable spicules, elongated rigid cuticular structures required for copulation, and the gubernaculum, a sclerotic cuticular structure that guides the spicules as they protract. The proctodeum, vas deferens, and intestine are connected to a common opening, the cloaca. Anterior to the cloaca is the hook, an external sclerotic structure with an associated sensillum, that functions in locating the hermaphrodite vulva. The fan is a cuticular structure produced by hypodermal cells, which contains nine pairs of sensory rays required to sense hermaphrodite contact.

Male tail cells required for particular mating behaviors have been identified by laser microsurgery experiments (Liu and Sternberg, 1995). The rays, sensory hook, and associated neurons are required for the early mating behaviors of backing, turning, and general vulva location. The spicules and their associated neurons and muscles are required, in part, for vulva location and for spicule insertion into the vulva prior to sperm transfer.

In this paper we describe the male-specific defects of *lin-29* mutants. We report that *lin-29* mutant males are capable of performing early mating behaviors including vulva location but are defective in the subsequent steps of spicule-mediated vulval attachment and sperm transfer. We describe the morphological basis of this defect, the temporal and spatial male-specific LIN-29 expression patterns, and the cell-type requirements for *lin-29(+)* with respect to spicule morphogenesis and male mating.

MATERIALS AND METHODS

Nematode Strains

The following alleles were used and are described by Hodgkin (1997) unless otherwise noted: LG I, *unc-13(e1091)* (Waterston and Brenner, 1978); *lin-28(n719);* LG II, *lin-4(e912); lin-29(n546, n836, ga94);* LG III, *ncl-1(e1865); unc-36(e251);* LG V, *him-5(e1467);* LG X, *lin-14(n179*ts). Integrated arrays containing the following reporter gene fusions were also used: *mab-18::lacZ* (S. Emmons, personal communication), *unc-4::lacZ* (Miller and Niemeyer, 1995), and *unc-5::lacZ* (J. Culotti, personal communication). An array containing the *lin-29(+)* fosmid H40L17 (*veEx112*, see below) was integrated into the genome by γ-ray irradiation. Two independent integrants were identified, *veIs17* and *veIs18*, and used in control experiments.

him-5 strains produce approximately 30% male progeny through an increased rate of non-disjunction of the X chromosome and were used as the wild-type control and genetic background in this work in order to obtain males easily. *him-5* males are morphologically indistinguishable from wild-type males. Strains were constructed by standard methods. Homozygosity of strains containing integrated *lacZ* fusion constructs was verified by assaying for expression in 10 or more progeny animals. *n546* and *n836* behave as null alleles of *lin-29* (Rougvie and Ambros, 1995). *ga94* is a hypomorphic allele (Bettinger *et al.*, 1997).

Indirect Immunofluorescence

Synchronized nematode cultures were fixed and antibody incubations were performed as described in Finney and Ruvkun (1990) with the modifications of Bettinger *et al.* (1996). The monoclonal antibody MH27 (Francis and Waterston, 1991) was kindly provided by the Waterston lab. The LIN-29 accumulation pattern in *him-5* hermaphrodites was indistinguishable from that of wild-type N2 hermaphrodites reported by Bettinger *et al.* (1996), and we did not detect LIN-29 accumulation in *lin-29(n546); him-5* or *lin-29(n836); him-5* males (data not shown). β-Galactosidase (β-gal) was detected with a mouse monoclonal antibody (Promega). In coimmunolocalization experiments, LIN-29 was detected using rhodamine-conjugated goat anti-rabbit secondary antibody, while β-gal was detected using fluoroscein-conjugated goat anti-mouse secondary antibody. Control experiments demonstrated that these secondary antibodies did not react cross-species to a detectable degree. In single-label experiments, LIN-29 was detected using a fluoroscein-conjugated goat anti-rabbit secondary. Affinity-purified secondary antibodies were obtained from Cappel.

Following ablation of particular cells, individual animals were staged and fixed as follows: To individual wells of a 96-well microtiter plate (Sarstedt) were added 89 μl of distilled H_2O, a worm to be fixed, 100 μl of 2× Ruvkun fixation buffer (Bettinger *et al.*, 1996), and 11 μl of 37% formaldehyde. The solutions were mixed and the plate was placed in a dry-ice/ethanol bath until frozen. The samples were thawed and carried through the fixation and incubation steps detailed in Bettinger *et al.* (1996). Supernatant was removed with a pipetman while observing the worm under a dissecting microscope.

Propidium iodide staining was performed as described by Euling and Ambros (1996). Confocal microscopy was performed with a Bio-Rad MRC 1024 system on a Nikon diaphot inverted microscope. Images were collected using a 60× PlanApo 1.4 NA lens.

Male Mating Assays

Wild-type and *him-5* male mating efficiency was assayed by placing a single L4-to-adult molt stage or very young adult male together with eight young adult *unc-13* hermaphrodites on a mating plate. To control for variation of mating efficiency due to age of plates (Liu and Sternberg, 1995), 30-mm agar plates were poured one day, seeded with a 5- to 7.5-mm lawn of *Escherichia coli* OP50 the next day, and used for mating assays the following day. The cross plates were placed at 20°C and monitored for non-Unc progeny 4 and 6 days after the crosses were initiated. *lin-29* mutant males were assayed in the same manner except that 8 to 10 *lin-29* L4 molt to young adult males and an equal number of young adult *unc-13* hermaphrodites were used in each mating. A control cross between 10 *him-5* males and 10 *unc-13* hermaphrodites produced 431 non-Unc and 435 Unc progeny. For the determination of the mating efficiency of postdauer males, dauer larvae were selected by treating starved cultures with 1% SDS for 30 min. The dauers were placed on seeded plates and allowed to develop at 20°C until the late L4 to young adult stage, when males were picked and assayed for mating efficiency.

Statistical analyses were performed using the *Z* test of significance. We observed a statistically significant difference between the mating frequencies of wild-type (N2) versus *him-5* males as reported previously by Hodgkin (1983), such that N2 males mated more efficiently than did *him-5* males. This result was observed regardless of whether the animals developed continuously or through the dauer larva stage (Table 1: $Z = 6.2$, $P = < 0.0001$; $Z = 3.3$, $P = 0.0003$).

Male mating behaviors were assayed by direct observation of cross plates containing individual males at 25–50× using a dissecting microscope at room temperature (21–23°C). Thirty *unc-13* adult hermaphrodites were evenly dispersed on a 5- to 7.5-mm-diameter *E. coli* lawn. One young adult male (≤4 h after exit from the fourth molt) was placed in the center of the lawn at $t = 0$. The male behaviors (Liu and Sternberg, 1995) were noted during a 20-min observation period using the following terminology: swim (no contact with hermaphrodite), hermaphrodite body contact, backing with tail contacting the hermaphrodite, turning of the tail around the circumference or an end of the hermaphrodite, forward movement of tail in contact with hermaphrodite, general vulva location movements, fine-tuning vulva location movements, and attachment of the tail to the vulva. While spicule insertion and sperm transfer were not always directly observed, vulva attachment was presumably an indication of spicule insertion. Plates were seeded 1–2 days prior to use and plates were closed during observation. If a male did not move for more than 30 s, the plate was tapped.

Cell Lineage, Male Tail, and Gonad Development and Spicule Protraction Analysis

Cell lineage analysis was performed using Nomarski optics at room temperature (22–23°C). B cell lineage divisions were observed in 16 *lin-29(n546)* males: four hatchlings were observed to the L1-to-L2 molt, three L1-to-L2 molt stage animals to the L2-to-L3 molt, and partial lineages were followed in nine L3-stage animals such that each cell division was observed at least three times.

Spicule length, ray formation, male gonad development, molt behavior, and developmental stage were assessed using Nomarski optics. L4-to-adult molt stage animals were identified by monitoring entry into lethargus and observing the old cuticle loosening around the head and sealing over the mouth (Singh and Sulston,

TABLE 1
Mating Ability of *lin*-29 and *him*-5 Males

Genotype	% males that mated (*n*)[a]
him-5	51 (234)[b,c]
him-5 PD[d]	82 (226)[b,e]
N2	85 (119)[b]
N2 PD[d]	95 (110)[b]
lin-29(n836); him-5; unc-36 ncl-1; veIs17[f]	30 (50)[b]
lin-29(n836); him-5	0 (308)[g]
lin-29(n546); him-5	0 (310)[g]
lin-29(n546); him-5 PD[d]	0 (300)[g]
lin-29(ga94); him-5	0 (200)[g]
lin-29(ga94); him-5 PD[d]	0.3 (300)[g,h]

[a] *n*, number of males tested.

[b] Single males were placed on a mating plate with eight *unc-13* hermaphrodites (see Materials and Methods). Four and 6 days later F1 progeny were scored for the Unc (self) and non-Unc (cross) progeny.

[c] This number is the average of two experiments: in one, 45/116 (38.7%) males mated; in the other, 74/118 (63.5%) males mated.

[d] PD, postdauer. The males used in the cross had developed through the dauer larval stage.

[e] This number is the average of two experiments: in one, 84/116 (72.4%) males mated; in the other, 102/110 (92.7%) males mated.

[f] The *lin-29(+)* extrachromosomal array *veEx112* was stably integrated into the genome of *lin-29(n836); him-5; unc-36 ncl-1* animals. Animals homozygous for the integrated array were scored for mating as described under Materials and Methods.

[g] Eight to 10 males of the indicated genotype were placed on a mating plate with 8 to 10 *unc-13* hermaphrodites (see Materials and Methods). Four and 6 days later F1 progeny were scored for Unc (self) and non-Unc (cross) progeny.

[h] On a single plate, three non-Unc progeny were found. We assume that these three animals were the product of a single mating. Each non-Unc animal produced Unc and Lin progeny, verifying that they were cross-progeny from a *lin-29(ga94)* male.

1978). Gonad development was judged relative to larval stage and molts.

Spicule protraction in animals under anesthesia was assessed by mounting males in 5 μl of M9 on 5% agar pads containing 5% (w/v) sodium azide (NaN_3). When the worms ceased moving they were observed using Nomarski optics. Under these conditions, 90% of wild-type ($n = 72$) and 94% of *him-5* ($n = 219$) spicules protracted.

Laser Killing of the B Cell

Cells were killed with a laser as described (Avery and Horvitz, 1987) except that the laser beam was transmitted via a fiber-optic cable (Bull's Eye ablation laser). Laser microsurgery was performed by direct observation of cell nuclei in anesthetized animals (mounted on 4–5% agar pads containing 5% NaN_3) using Nomarski optics. B cell progeny were eliminated by killing the undivided B blast cell during the early L1 stage or by killing B.a and B.p in the late L1 stage. Operated animals were remounted and their tails were examined to ensure proper destruction of the B cell(s) prior to fixation for immunolocalization studies.

Mosaic Analysis

Mosaic analysis was performed by scoring animals of the genotype *lin-29(n836); ncl-1 unc-36; him-5; veEx112* for mitotic loss of the extrachromosomal array, *veEx112* (Bettinger *et al.*, 1997). *veEx112* carries *lin-29(+)* on fosmid H40L17, *ncl-1(+)* on cosmid C33C3 (Miller *et al.*, 1996), and *unc-36(+)* on plasmid R1p16 (Herman *et al.*, 1995). *veEx112* fully rescues the *lin-29(0)* phenotype in hermaphrodites and males (Bettinger *et al.*, 1997, and data not shown). Furthermore, LIN-29 accumulation in two independent integrants of *veEx112* in the genetic background used for mosaic analysis [*lin-29(n836); ncl-1 unc-36; him-5; veIs17* and *lin-29(n836); ncl-1 unc-36; him-5; veIs18*] was indistinguishable from the wild type (data not shown). These strains are rescued for the *lin-29* phenotype (Table 1 and Table 4).

Male animals were identified and individual cells were scored for the *ncl-1* phenotype (Ncl) during the late L2 or early L3 stage. The animals were scored for tail morphology, spicule length and protraction, and, when applicable, mating ability, during the adult stage.

RESULTS

lin-29 Mutant Males Are Mating Defective but Perform Early Mating Behaviors

We tested the mating efficiency of *lin-29* mutant males by scoring for cross-progeny from matings between *lin-29* males and uncoordinated (Unc) hermaphrodites (see Materials and Methods). Unc hermaphrodites were used in these assays because greater mating efficiencies are observed when hermaphrodites are unable to move (Hodgkin, 1983). *lin-29(n546); him-5* and *lin-29(n836); him-5* males failed to mate with *unc-13* hermaphrodites as was indicated by the absence of non-Unc progeny (Table 1). In contrast, 51% of *him-5* males mated successfully with *unc-13* hermaphrodites as measured by production of non-Unc progeny in control crosses. A *lin-29(+)*-containing fosmid clone (Bettinger *et al.*, 1997) rescued the male mating defect of *lin-29* mutant males (Table 1), further demonstrating the *lin-29* dependence of this phenotype.

We tested whether *lin-29* mutant males are defective in any of the five defined steps of male mating behavior (Liu and Sternberg, 1995) in order to identify more specifically the defect(s) caused by *lin-29* loss of function. We observed *lin-29* males in mating tests with *unc-13* hermaphrodites and found that they respond to hermaphrodite contact by backing, and they also perform the turning and vulva location behaviors, although at a lower frequency (Table 2) and with less coordination than *him-5* males. On average, *lin-29* mutant males spent less time in contact with hermaphrodites and performed mating behaviors more slowly than did *him-5* males.

Since *lin-29* males respond to hermaphrodites by performing the early mating behaviors, the functions of the sensory rays, the hook, and associated sensilla are not inactivated by *lin-29* mutation. Consistent with this observation, the hook appears properly formed in *lin-29; him-5* adults, and the fan contains the normal number of rays. The

TABLE 2

Ability of *him-5* and *lin-29* Mutant Males to Perform Mating Behaviors

Behavior[a]	Percentage of males that performed the behavior		
	him-5	*lin-29(n546); him-5*	*lin-29(ga94); him-5*
Contact	100	100	100
Backing	100	97	93
Turning[b]	80	20	67
Vulva location			
General	77	23	53
Fine-tuning	67	7[c]	13[c]
Vulval attachment[d]	63	0	0

Note. The percentages represent the numbers of males that performed the behavior at least once during the 20-min observation period. $n = 30$ for each genotype.

[a] The behaviors listed are adapted from Liu and Sternberg (1995) and are further defined under Materials and Methods.

[b] The number includes males that turned either circumferentially around the width or at the end of a hermaphrodite. Turns were not counted if the male performed the ventral arch but failed to maintain contact with the hermaphrodite.

[c] Some of the males that located the vulva protracted their spicules, but did not insert them into the vulva. The protracted spicules appeared flattened, lacking the rigidity characteristic of wild-type spicules.

[d] Vulva attachment was defined as a male tail attached to the hermaphrodite vulva without shifting position for greater than 5 s. This behavior was used as an indicator of spicule insertion.

rays, as judged by relative position, size, and shape, appear essentially normal (data not shown). We note that ray formation in *lin-29* mutants is slightly delayed relative to *him-5* males (Table 3), but is nevertheless complete by the end of the fourth molt. The observed delay is unlikely to contribute to the mating defect, since mating behaviors are not initiated until the L4 stage cuticle is shed (Hodgkin, 1983).

We tested whether *lin-29(ga94),* a hypomorphic allele (Bettinger *et al.*, 1997), produces defects in males. In contrast to animals bearing null mutations in *lin-29* [denoted *lin-29(0)*], *lin-29(ga94)* hermaphrodites and males form adult-type cuticle at the fourth molt (Bettinger *et al.*, 1997, and data not shown). In hermaphrodites, the *lin-29(ga94)* allele causes the majority of animals to be egg-laying defective and have vulval protrusions similar to animals bearing *lin-29(0)* alleles. We found that although *lin-29(ga94)* males perform the backing, turning, and vulval location behaviors with an increased frequency relative to *lin-29(0)* animals (Table 2), they are nevertheless unable to mate (Table 1).

Unlike in *lin-29(0)* hermaphrodites, the egg-laying defect of *lin-29(ga94)* hermaphrodites is usually suppressed by development through the dauer larval stage (Bettinger *et al.*, 1997). Therefore, we tested whether the male mating defect of *lin-29(ga94)* postdauer males is similarly suppressed. We found that the mating efficiency of *lin-29(ga94)* males was essentially unaltered by development through the dauer larva stage (Table 1). Interestingly, in control experiments, we found that development through the dauer larva stage increased the mating efficiency of both wild-type and *him-5* males (Table 1) in a statistically significant manner ($Z =$

TABLE 3

Ray Formation and Gonad Migration in *him-5* and *lin-29* Mutant Males

Genotype	Percentage of males with delayed ray extension (n)[a]	Percentage of males with abnormal gonad migration (n)[d]
him-5	0 (43)[b]	0 (47)
lin-29(n546); him-5	41 (42)[c]	20 (46)[e]
lin-29(n836); him-5	44 (41)[c]	40 (43)[f]
lin-29(ga94); him-5	21 (42)[c]	40 (38)[g]

[a] Ray formation was examined in animals in the fourth molt stage. Ray extension was considered delayed if rays on one side of the animal had no extension or were extended less than approximately 25% of wild-type full length. *n,* number of animals; one side of the tail was examined per animal.

[b] Thirty-four animals had fully extended rays and 9 animals had rays extended 50–75% of wild-type full length.

[c] Of the animals exhibiting delayed ray formation, ray length ranged from 0 to 25% full-length ray process extension.

[d] The gonad migration patterns of late L4 or fourth molt stage males were observed and scored for size, extent of migration, position, and number of turns. Representative migration patterns are shown in Fig. 4.

[e] The gonad phenotypes of the nine *lin-29(n546); him-5* males with defects were as follows: one L4-to-adult molt animal had a retarded gonad resembling an L3-to-L4 molt stage gonad (Fig. 4B), one made the ventral turn late (Fig. 4C), one turned anteriorly rather than posteriorly following the ventral turn (Fig. 4D), two failed to migrate dorsally and instead made a 180° turn on the ventral side prior to migrating posteriorly (Fig. 4E), one failed to make the dorsal turn and continued to migrate anteriorly into the head (Fig. 4F), one made additional abnormal turns (e.g., Fig. 4G), and two made additional abnormal turn(s) with incomplete extension.

[f] The gonad phenotypes of the 17 *lin-29(n836); him-5* males with defects were as follows: five L4-to-adult molt animals had retarded gonads resembling L3-to-L4 molt stage gonads (e.g., Fig. 4B), three made additional abnormal turn(s) with incomplete extension, one made the ventral turn late (Fig. 4C), one failed to make the ventral turn and made additional abnormal turns, four made additional turns (e.g., Fig. 4G), and three failed to migrate dorsally and instead made a 180° turn on the ventral side (Fig. 4E); two of these latter animals had incomplete posterior migration.

[g] The gonad phenotypes of the 15 *lin-29(ga94); him-5* males with defects were as follows: three L4-to-adult molt animals had retarded gonads resembling the L3 or L3-to-L4 molt stage (e.g., Fig. 4B), nine made additional abnormal turns (e.g., Fig. 4G), and three made additional abnormal turn(s) with incomplete extension.

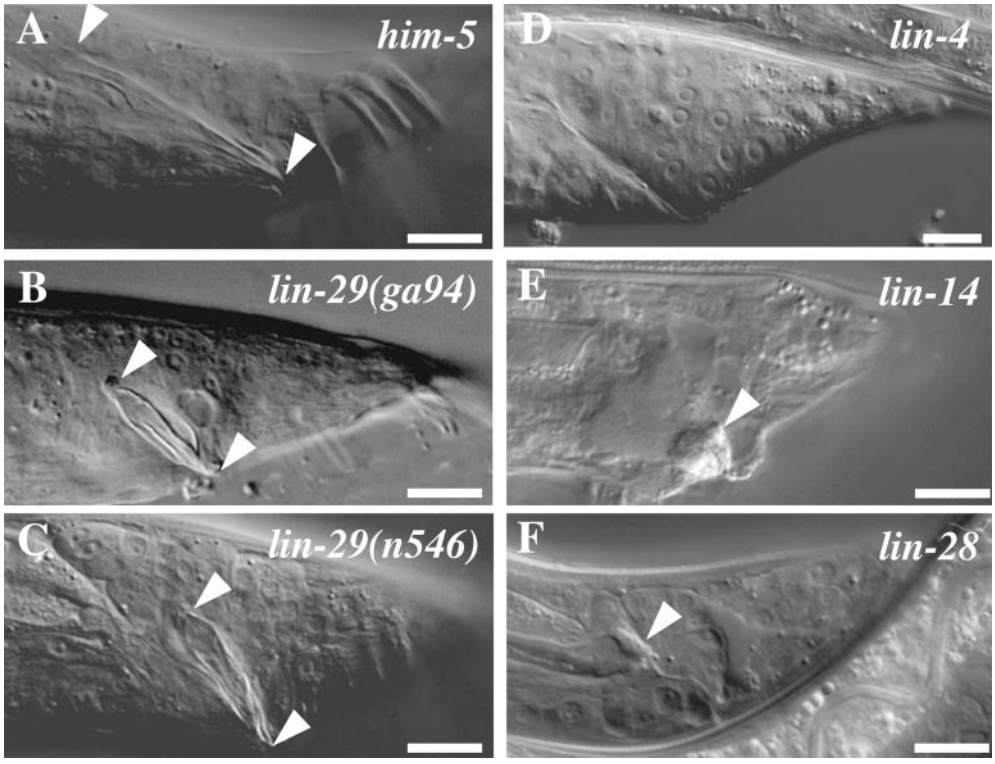

FIG. 2. Spicule phenotypes of adult wild-type and heterochronic mutant males. (A) *him-5*. (B) *lin-29(ga94); him-5*. (C) *lin-29(n546); him-5*. (D) *lin-4; him-5*. Because *lin-4; him-5* males do not produce obvious male tail structures, gonad migration patterns were used to identify males. (E) *lin-14*(ts)*; him-5* grown at 25°C. (F) *lin-28; him-5*. Arrowheads in A–C indicate the two ends of the spicules to show length. Arrowheads in E and F indicate highly refractile, spicule-like material that is typical of *lin-14; him-5* and *lin-28; him-5* males. Scale bars, 10 μm.

2.5, $P = 0.006$ and $Z = 7.0$, $P = < 0.0001$, respectively; and see Materials and Methods).

In contrast to the earlier mating steps, *lin-29* mutant males did not exhibit vulva attachment via spicule insertion (Table 2). Since attachment to the vulva by spicule insertion is mediated by the spicule cells and their associated neurons and muscles (Liu and Sternberg, 1995), this observation suggests a defect in one or more of these cell types.

lin-29 Mutant Males Have Short Spicules

To determine whether spicules appear defective in *lin-29* mutants, we examined spicule length and morphology in adult male tails using Nomarski optics. We observed short spicules in all *lin-29* mutant animals examined ($n = 242$; Figs. 2A–2C; Table 4). In contrast to the short spicule phenotype observed in mutants with defects in B cell fate specification (Chamberlin and Sternberg, 1993) or in animals in which the M blast cell has been eliminated through laser microsurgery (Sulston *et al.*, 1980), *lin-29* spicules rarely appear crumpled (Table 4, and see legend). We quantitated spicule length and found that spicules in *lin-29* mutant adults are, on average, 43% shorter than in *him-5* males (Figs. 2A–2C; Table 4). Spicule length did not vary significantly between *lin-29(0)* and *lin-29(ga94)* animals (Figs. 2B and 2C) nor was it altered by development through the dauer larva stage (Table 4). The spicule length defect was rescued by a *lin-29(+)*-containing fosmid clone (Table 4).

Each spicule is composed of four structural cells which are likely to be responsible for secretion of the rigid cuticular coating, two neurons, and two sheath cells (see Emmons and Sternberg, 1997, for review). Because these cells are all derived from the male-specific B blast cell (Sulston *et al.*, 1980), we examined *lin-29(0)* males for a B cell lineage defect. The B cell lineage was followed in *lin-29(n546)* males and appeared wild-type in cell lineage pattern and cell division timing (data not shown). Spicule development was also monitored following the completion of cell divisions, from the third through the fourth molt, and appeared similar to that in the wild type. The short morphology of the *lin-29* spicules was not obvious until after the fourth molt, and it appears to result from defective spicule elongation.

TABLE 4
Spicule Length of Wild-Type, *him-5,* and *lin-29* Mutant Males

Genotype	% short spicules (*n*)[a]	Spicule length (μm)[b]
him-5	0 (64)[c]	42.2 ± 3.4
him-5 PD[d]	ND	42.7 ± 3.4
N2	ND	42.4 ± 1.8
N2 PD[d]	ND	39.9 ± 2.7
lin-29(n836); him-5; unc-36 ncl-1; vels17	ND	43.7 ± 2.9
lin-29(ga94); him-5	100 (57)[e]	24.9 ± 4.5
lin-29(ga94); him-5 PD[d]	ND	23.0 ± 3.9
lin-29(n546); him-5	100 (53)[f]	23.3 ± 4.4
lin-29(n546); him-5 PD[d]	ND	25.2 ± 3.8
lin-29(n836); him-5	100 (52)[g]	ND

[a] Animals were examined in Nomarski optics and spicule length was judged by eye.

[b] Spicule length was measured in photo-micrographs of young adult animals. The mean spicule length for each genotype was determined from 20 spicule length measurements, in which one spicule was measured in each of 20 animals (see Materials and Methods).

[c] The two spicules in each *him-5* male were of similar length. No *him-5* spicules appeared as short as any *lin-29* mutant spicules.

[d] PD, postdauer. These males had developed through the dauer larval stage.

[e] Six animals had spicules slightly longer than average, approximately 75% of wild-type length. Two animals had spicules of unequal length, one approximately 50% and the other approximately 75% of wild-type length.

[f] One animal had significantly shorter spicules, approximately 25% of wild-type length. Eight animals had spicules of different lengths, one approximately 50% of wild-type length and one approximately 25% of wild-type length and crumpled. One animal had spicules approximately 75% of wild-type length.

[g] Two animals had short, crumpled spicules approximately 25% of wild-type length. Five other animals had spicules 50% of wild-type length, one crumpled and one not crumpled.

lin-4, lin-14, and lin-28 Mutants Have Severe Spicule Defects

We tested whether mutations in the heterochronic genes *lin-4*, *lin-14*, and *lin-28* also cause spicule defects, because these genes control the stage specificity of LIN-29 accumulation in the hypodermis and in the hermaphrodite vulva (Bettinger *et al.*, 1996). *lin-4; him-5* mutant males produce no obvious spicules (Fig. 2D) and they lack the refractive yellow material that appears to correlate with spicule socket cell differentiation (L. Jiang and P. Sternberg, personal communication). *lin-14; him-5* and *lin-28; him-5* males have malformed spicules with very abnormal morphology, containing a variable amount of yellow material in the position where spicules normally form (Figs. 2E and 2F). Thus, the heterochronic genes *lin-4*, *lin-14*, *lin-28*, and *lin-29* are all required for normal spicule formation.

LIN-29 Temporal Expression in Wild-Type Males

We used antibodies raised against LIN-29 (Bettinger *et al.*, 1996) in whole-mount immunolocalization experiments to determine the temporal and spatial patterns of LIN-29 accumulation in males. We found that non-sex-specific LIN-29 accumulation in *him-5* males is similar to that in hermaphrodites (Bettinger *et al.*, 1996): LIN-29 accumulates in the nuclei of all pharyngeal cells beginning during the L1 stage, in a few head neurons beginning during the L2 stage, and throughout the majority of the hypodermis in the L4 stage (data not shown and Fig. 3D). The male-specific patterns of LIN-29 accumulation are described in the following sections.

LIN-29 accumulates in the linker cell. The first male-specific LIN-29 accumulation is detected in the nucleus of the linker cell (LC) positioned at the tip of the growing end of the gonad (Fig. 3A). During male gonadogenesis, the migration of the LC directs the growth path of the single gonad arm as diagrammed in Fig. 4A (Kimble and Hirsh, 1979). The LC migrates anteriorly along the ventral side of the animal during the late L1 and L2 stages and then completes a 180° turn during the L2-to-L3 molt, first turning dorsally and then posteriorly. It then migrates posteriorly along the dorsal side of the animal until the nongrowing end of the gonad is reached in the mid-to-late L3 stage. The LC then crosses back to the ventral side of the animal and continues migrating posteriorly to the male tail, where it is induced to undergo programmed cell death producing a connection between the vas deferens and the cloaca (Kimble and Hirsh, 1979). LIN-29 accumulates in the LC during the L3 stage (Fig. 3A), after the gonad arm has completed the 180° turn. LIN-29 remains detectable until the late L4 stage when its disappearance is presumably due to LC destruction.

We examined *lin-29* mutant males for gonad migration defects that could reflect a function for LIN-29 in the linker cell. We found that *lin-29(0)* and *lin-29(ga94)* males exhibit defects in gonad migration (Table 3 and see legend; Figs. 4B–4G), albeit with incomplete penetrance (20–40%). The gonad migration phenotypes observed included failure to execute the 180° turn, execution of extra turns, and delayed extension of the gonad arm.

LIN-29 accumulates in ventral cord nuclei. During the late L3 stage, five to seven nuclei that belong to the preanal ganglion accumulate LIN-29. This identification was confirmed by the colocalization of LIN-29 and β-galactosidase (β-gal) in a *mab-18::lacZ; him-5* strain (S. Emmons, personal communication; Zhang and Emmons, 1995). Four of five *mab-18::lacZ*-expressing preanal ganglion nuclei also accumulate LIN-29 and were identified by position as the CA9, CP9, AS11, and VA11 neurons which are descended from P11.a. LIN-29 accumulation in the preanal ganglion cluster persists through adulthood (Figs. 3B, 3C, and 3F).

In late L4 stage and adult males, additional ventral cord nuclei anterior to the preanal ganglion accumulate LIN-29 (Figs. 3B and 3C). Although LIN-29-accumulating nuclei in

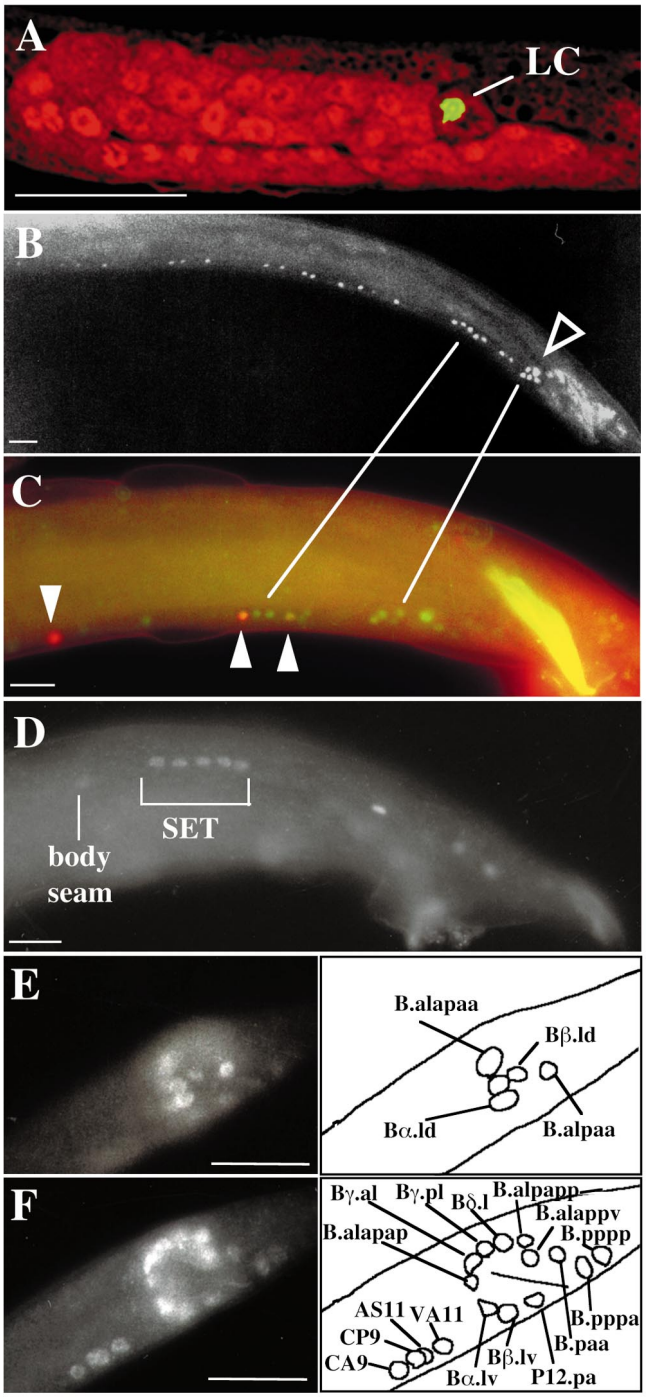

FIG. 3. LIN-29 accumulation in wild-type males. (A) A *him-5(e1467)* L3 stage male stained with anti-LIN-29 (green) and propidium iodide (red) to show nuclei. LIN-29 accumulates in the linker cell (LC) nucleus. (B) A *him-5* adult male stained with anti-LIN-29 antibody, showing accumulation of LIN-29 in some nuclei of the ventral cord, including nuclei of the preanal ganglion (open arrowhead). (C) An adult male bearing a *unc-4::lacZ* fusion costained with antibodies to β-gal and LIN-29 (see Materials and Methods). The photograph shown is a merged image of both signals. Nuclei that accumulate only LIN-29 appear green, whereas nuclei containing both LIN-29 and β-gal appear orange (arrowheads). Lines indicate corresponding regions of the animal shown in B. (D) Left lateral view of a young adult *him-5* male. The anteriorly migrating SET nuclei accumulate LIN-29. (E) Left panel, left lateral view of an L4 stage *him-5* male stained with anti-LIN-29 antibody. Right panel, a tracing of the same animal indicating identities of LIN-29-accumulating nuclei. (F) Left panel, an internal focal plane of the animal shown in E. Right panel, a tracing indicating the cell identities of LIN-29-accumulating nuclei. Scale bars, 10 μm.

the ventral cord have not been unambiguously identified, the size and positions of these nuclei together with analysis of animals containing reporter fusion genes suggest that they include VA and DA motor neurons. Males bearing a *unc-4::lacZ* fusion costained with anti-LIN-29 and anti-β-gal antibodies revealed overlapping patterns of accumulation (Fig. 3C), whereas β-gal and LIN-29 appeared nonoverlapping in ventral cord nuclei in a strain bearing a *unc-5::lacZ* fusion (data not shown). *unc-4::lacZ* is expressed in the VA and DA motor neurons (Miller and Niemeyer, 1995; D. Miller, personal communication) and *unc-5::lacZ* is expressed in VD and DD motor neurons of adult hermaphrodites (J. Culotti, personal communication). Assuming that these reporter fusions are expressed with the same cell-type specificity in the ventral cord of both sexes, these results suggest that LIN-29 accumulates in VA and/or DA motor neurons. The VA and DA motor neurons are responsible, in part, for backward movement (Chalfie *et al.*, 1985). *lin-29* mutants do not have an obvious defect in backward movement; however, *lin-29* males do exhibit a reduction in their ability to perform the backing and turning mating behaviors (Table 2), which are both backward movements. The accumulation of LIN-29 in ventral cord neurons is not observed in hermaphrodites. Also in contrast to hermaphrodites, the ventral hypodermal nuclei positioned within the ventral cord do not contain detectable levels of LIN-29 in adult males (data not shown).

LIN-29 accumulates in the male tail seam, but is not required for its fusion. *lin-29* is required for terminal differentiation of the lateral hypodermal seam cells during the L4-to-adult molt in both hermaphrodites (Ambros and Horvitz, 1984) and males (compare Figs. 5C and 5D). Seam cell terminal differentiation is characterized by the cessation of cell divisions, the fusion of the seam cells into bilateral syncytia, and the synthesis of an adult cuticle containing alae, cuticular ridges that extend laterally over the seam (Ambros and Horvitz, 1984). On each side of the male tail, the most posterior lateral seam cells, generated by the V5 and V6 blast cells, fuse into a syncytium known as the male tail seam or SET. Each SET is separated from the corresponding lateral seam of the main body by a desmosome and differs from the main body seam in that it lacks adult alae (Sulston *et al.*, 1980).

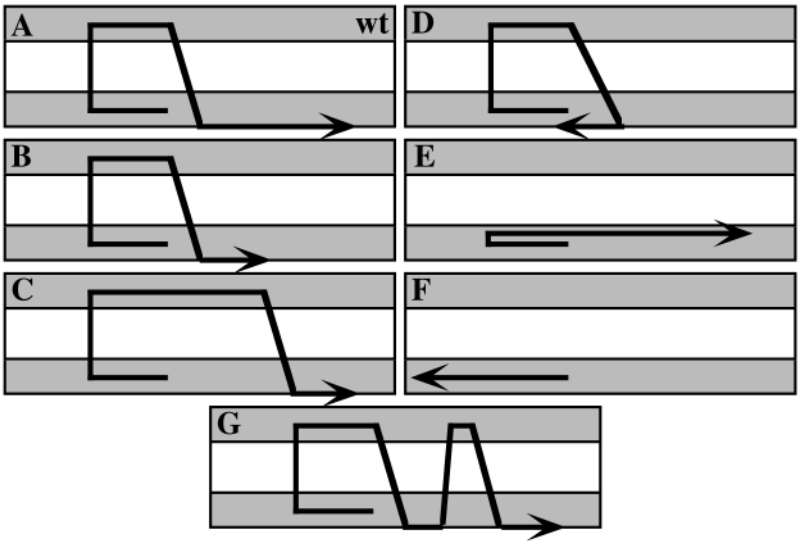

FIG. 4. Representative abnormal gonad migrations observed in *lin-29* mutant males. In each panel anterior is to the left. The shaded boxes represent the dorsal (top) and ventral (bottom) body walls. Only the left body wall is shown (white box). The black arrow indicates the path of the migrating LC. Adapted from Antebi *et al.* (1998). (A) Wild-type. (B–G) Representative *lin-29* mutants.

LIN-29 accumulates in the lateral body seam cell nuclei and in the SET nuclei in L4 stage wild-type males (Fig. 3D). We tested whether *lin-29* activity is required for SET cell fusion during the fourth larval stage by using the MH27 antibody to monitor cell fusions during SET formation. The MH27 antibody recognizes an antigen in adherens junctions and effectively outlines hypodermal cells (Francis and Waterston, 1991). We compared the MH27 staining in *him-5* males to *lin-29(n546); him-5* males and found that the SET was unfused during the L3 stage in each case (Figs. 5A and 5B). During the fourth molt the lateral seam cells of the main body fused in *him-5* males but continued to divide and remained unfused in *lin-29(n546); him-5* mutants (Figs. 5C and 5D). In contrast, the SET fused normally in both *him-5* and *lin-29(n546); him-5* males (Figs. 5C and 5D), and the SET nuclei migrated anteriorly as in wild-type animals (Sulston *et al.*, 1980). Similar results were observed in *lin-29(n836); him-5* animals (data not shown). These results indicate that the fusion of the male tail hypodermal seam, unlike that of the lateral hypodermal seam (Ambros and Horvitz, 1984; Bettinger *et al.*, 1997), does not require LIN-29.

LIN-29 accumulates in B cell progeny. The spicule cells are all derived from the B.a lineage. The fate of B.a progeny, and therefore the final structure of the spicules, is dependent on a variety of cell types, including F, U, and Y progeny (Chamberlin and Sternberg, 1993). In addition, ablation of the M blast cell in males produces short spicules with a crumpled morphology, indicating that the sex muscle cells are required for spicule elongation (Sulston *et al.*, 1980). In contrast to the LIN-29 accumulation pattern observed in wild-type hermaphrodites (Bettinger *et al.*, 1996), LIN-29 is not detectable in the F, U, Y, or M blast cell nuclei, or in their progeny, in males. LIN-29 is detected in the progeny of B (Figs. 3E and 3F).

LIN-29 is first detectable in B cell progeny during the L3 stage. Although all B.a and B.p progeny accumulate LIN-29 (Figs. 3E and 3F), the LIN-29 accumulation signals appear weaker and transient in B.p progeny. The identification of these LIN-29-accumulating cells as B progeny is based on their size, shape, and relative position within the male tail (Sulston *et al.*, 1980) and is further supported by laser microsurgery experiments and the examination of LIN-29-accumulation patterns in a mutant with defects in B cell specification. Elimination of the B cell by laser microsurgery during the L1 stage (n = 11; see Materials and Methods) reduced the number of LIN-29-accumulating nuclei in the male tail by 10–15 (data not shown). The number of LIN-29-accumulating nuclei was also reduced (by 7–11 nuclei) in *mab-9* mutant males (data not shown), which exhibit a B to Y cell fate transformation (Chisholm and Hodgkin, 1989). LIN-29 appears to persist in B.a progeny in the adult stage; however, the extensive cell movements that occur in the late L4 stage male tail (Sulston *et al.*, 1980) have precluded the precise identification of LIN-29 staining cells in adult male tails.

lin-29 Function Is Required in the AB.pr Lineage for Spicule Length and Mating

We used mosaic analysis to determine in which cell lineages *lin-29(+)* is required in the developing male tail for proper spicule length and mating. We scored for the mitotic loss of a *lin-29*-rescuing extrachromosomal array to identify

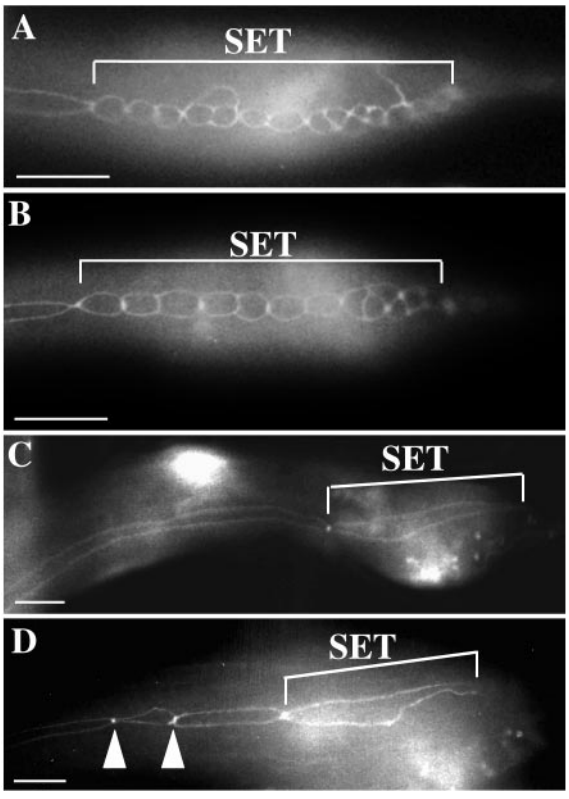

FIG. 5. Fusion of male tail seam (SET) cells. Fixed animals were stained with the MH27 antibody to visualize SET cell junctions (see Materials and Methods). Left lateral views of male tails are shown. The SET is indicated by a bracket and is unfused in A and B and fused in C and D. The open arrowheads indicate the junctions of the lateral body seam and the SET. Scale bars, 10 μm. (A) *him-5* L3 stage. (B) *lin-29(n546); him-5* L3 stage. The minor difference in the pattern of the SET cells reflects a slight difference in the age of the animals in A and B. (C) *him-5* adult. Both the lateral seam and the SET have fused. (D) *lin-29(n546); him-5* adult. Note that the lateral seam of the main body remains unfused, while the SET fuses normally. Solid arrowheads indicate seam cell junctions.

clones of *lin-29(−)* cells in animals that were otherwise *lin-29(+)*. Cells in *lin-29; ncl-1 unc-36* animals transgenic for an extrachromosomal array carrying wild-type copies of *lin-29*, *ncl-1* and *unc-36* were scored for the Ncl phenotype, which is cell-autonomous and scorable in most cell types (Hedgecock and Herman, 1995), to identify cells which had lost the array and were thus *lin-29(−)*. The extrachromosomal array carries *unc-36(+)* to aid in the identification of certain classes of mosaic animals. Because the focus of *unc-36* activity is in the AB.p lineage (Kenyon, 1986), animals that lose the array early in the AB.p lineage are Unc and therefore are impaired for mating. Unc animals were scored for spicule length but were not assessed for a mating defect associated with loss of *lin-29(+)* (see legend to Fig. 6A).

We identified five mosaic animals that had lost the array in P1 while maintaining it in AB. All five of these animals had spicules of wild-type length (Fig. 6A), indicating that the focus of *lin-29* activity for spicule length is within the AB lineage. Mosaic animals with losses in AB are consistent with this observation. We identified four animals that had lost the array in AB while maintaining it in P1 (Fig. 6A), and all of these animals had short spicules. We identified animals that had lost the array at later cell divisions within the AB lineage to determine more precisely the focus of *lin-29(+)* activity. The AB.a lineage gives rise to most of the main body and lateral hypodermis, which accumulate LIN-29 and in which *lin-29(+)* is required for terminal differentiation (Bettinger *et al.*, 1996, 1997). AB.a also gives rise to the V6 blast cells that generate rays 2–6 and contribute to the SET. Two of two animals that had lost the array in AB.a had spicules of wild-type length (Fig. 6A), indicating that the ray and hypodermal cells derived from AB.a do not play essential *lin-29*-dependent roles in spicule morphogenesis. In contrast, all six animals with losses in AB.p or AB.pr had short spicules (Fig. 6A), indicating that *lin-29(+)* is required within the AB.pr lineage for wild-type spicule length. Of these, the three animals with losses in AB.pr were non-Unc and failed to mate, suggesting that wild-type-length spicules are required for mating.

The bilateral blast cell pairs V5, T, and P11 originate from AB.pla and AB.pra (Fig. 6A). All nine mosaics with losses in AB.pl, AB.pla, or AB.pra developed wild-type-length spicules (Fig. 6A). These mosaic animals suggest that wild-type spicule length does not require *lin-29(+)* in the progeny of the male blast cells V5 and T that generate rays 1 and 7–9 and hypodermal cells or in the progeny of P11 that generate cells of the preanal ganglion. However, since we were unable to find a complex mosaic with a double loss in AB.pla and AB.pra, we were unable to assess the consequence of bilateral losses in these lineages.

AB.plp generates two blast cells, F and U, known to be required for specification of spicule cell identity (Chamberlin and Sternberg, 1993) and that generate male-specific neurons (Sulston *et al.*, 1980). Proper spicule length does not require *lin-29(+)* in these cells as three of three animals with array loss in AB.plp and three of three animals with losses in AB.pl had wild-type-length spicules. These mosaics further suggest that the AB requirement for *lin-29(+)* is within the AB.prp lineage (Fig. 6A).

Four of six animals with losses in AB.prp had short spicules consistent with a requirement for *lin-29(+)* within the AB.prp cell lineage. However, the remaining two AB.prp losses had spicules of normal length, indicating that this requirement for *lin-29(+)* is not absolute. AB.prp gives rise to the B and Y male-specific blast cells. Four animals with losses later in the AB.prp lineage that resulted in array loss from Y but not from B had wild-type-length spicules and two of these mated. These four mosaic animals indicate that *lin-29(+)* is not required in the postcloacal sensilla

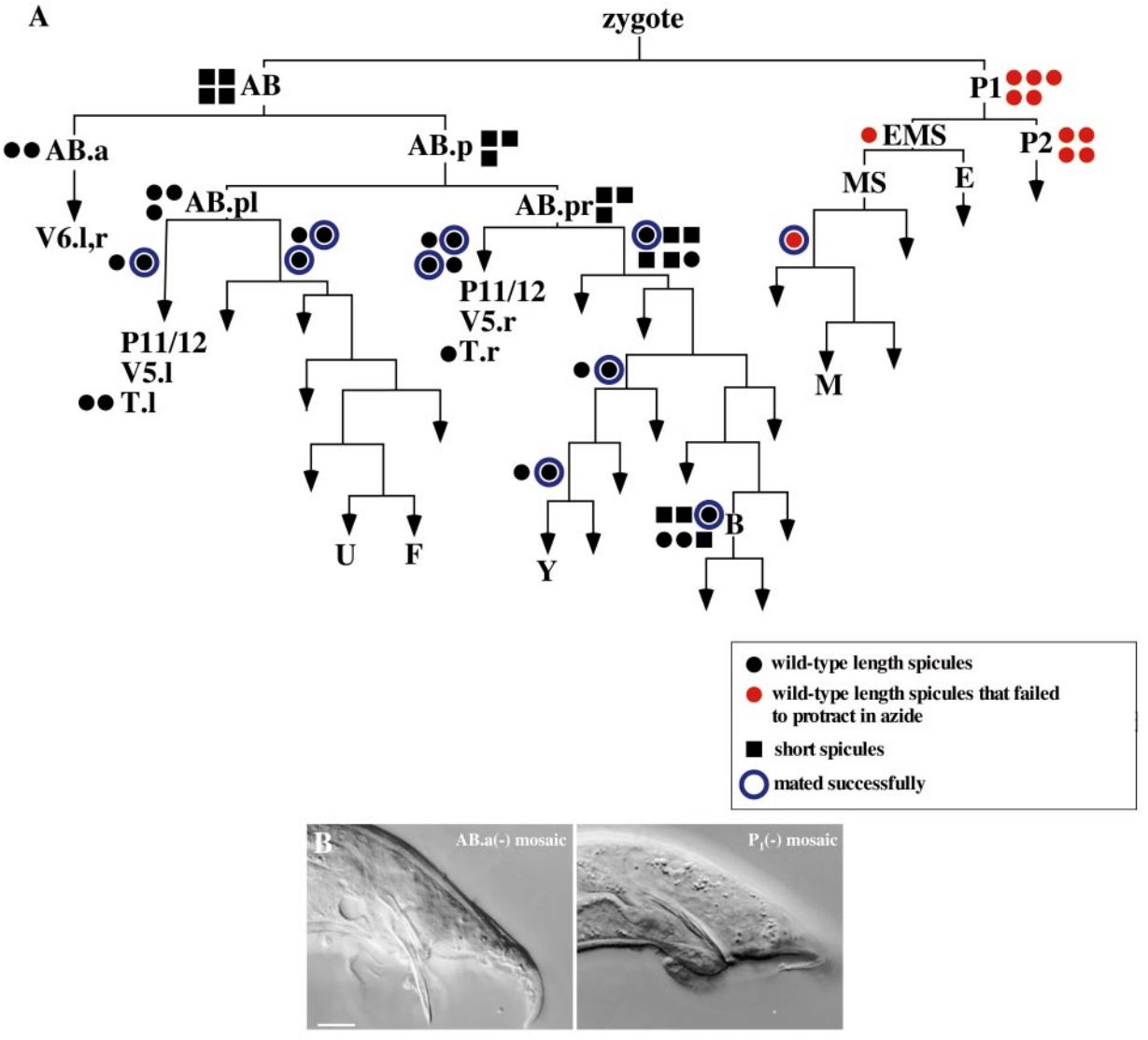

FIG. 6. Analysis of *lin-29* male mosaics. (A) An abbreviated lineage is shown indicating the origins of male-specific blast cells, several of which are known to be important for spicule development (Sulston and Horvitz, 1977; see text). Vertical lines indicate individual cells and horizontal lines indicate cell divisions. The positions of the symbols indicate where array loss occurred in an individual animal. Filled circles indicate animals with wild-type-length spicules. Red circles indicate animals with wild-type-length spicules that failed to protract in azide. Filled squares indicate animals with short spicules (see Materials and Methods). Blue outlines indicate that the animal mated successfully with *unc-13* hermaphrodites, producing non-Unc progeny. The four AB and three AB.p mosaics were Unc and not tested for mating. All of the remaining animals were tested for mating. No animals with short spicules produced cross-progeny. The mosaic animal indicated as an EMS loss may have been an MS loss as the Ncl phenotype is not scorable in the E cell lineage, which gives rise solely to gut cells. (B) Spicule protraction of *lin-29* mosaics. Nomarski photomicrographs of representative anesthetized *lin-29*-mosaic adult males are shown. Spicules of mosaics with losses in the AB lineage that were wild-type in length protracted in azide (left), whereas spicules of P1(−) mosaics failed to protract in azide (right).

generated by Y. In contrast, three of six animals with simple B losses developed short spicules. Together, these results suggest a requirement for *lin-29(+)* in the AB.pr lineage for proper spicule length and mating that traces, in part, to the B cell lineage.

A Role for lin-29(+) in the P1 Lineage

All five animals with P1 losses had a subtle defect. When wild-type or *him-5* males are anesthetized in 5% sodium azide, their spicules protract from the gubernaculum (see

Materials and Methods). The spicules of mosaic animals with P1 losses, although wild-type in length, failed to protract when the animals were anesthetized in azide (Fig. 6B), and when recovered from anesthesia these animals failed to mate (Fig. 6A). The failure of the spicules to protract was observed in all animals with losses within the P1 lineage ($n = 11$), but was not observed in mosaic animals with losses within the AB lineage which had wild-type-length spicules ($n = 26$; animals with short spicules cannot be scored for this phenotype). The observed spicule protraction defect was due to loss of the *lin-29(+)* array since the spicules of control animals, which maintained the array stably, protracted in azide ($n = 25$). Furthermore, the spicules of *unc-36 ncl-1; him-5* mutant males protract in azide indicating that this defect is due to loss of *lin-29(+)*. The failure to mate of 10/11 animals with losses in the P1 lineage is not a consequence of the anesthesia because approximately 41% of AB(−) mosaics with wild-type-length spicules and 50% of anesthetized *him-5* males ($n = 30$) mated when recovered from azide.

DISCUSSION

A Role for LIN-29 in Spicule Morphogenesis

We show that *lin-29* plays a role in the morphogenesis of the specialized male tail used in mating. *lin-29* mutant males can perform early mating behaviors, but they do not perform vulval attachment via spicule insertion and, as a consequence, are unable to mate (Tables 1 and 2). *lin-29* mutant males have abnormally short spicules (Table 4), but do not appear to have a cell division defect in the B cell lineage which generates the spicules, nor do *lin-29* mutants appear to have defects in overall tail structure prior to exit from the fourth molt. Rather, the *lin-29* spicule defect becomes apparent as the animal emerges from the fourth molt.

Immunolocalization experiments revealed that LIN-29 accumulates in B cell progeny during the third larval stage in wild-type animals. Because B.a progeny cells generate the spicules, these cells could represent the site of action of *lin-29* with respect to spicule length. Indeed, analysis of genetic mosaics reveals a major requirement for *lin-29(+)* in the B cell lineage (Fig. 6). However, mosaic analysis also suggests that *lin-29(+)* function in the B cell lineage is not the sole focus of *lin-29* activity with respect to spicule length since 33% of AB.prp losses and 50% of simple B cell losses were wild-type for spicule length. Thus, although the requirement for *lin-29(+)* function traces to the AB.pr lineage, the lineage which eventually gives rise to the B cell, the focus of *lin-29(+)* within AB.pr does not trace exclusively to the B cell lineage.

The incomplete requirement for *lin-29(+)* in the B cell lineage is unlikely to result from perdurance of LIN-29 after the B lineage has lost the extrachromosomal array. LIN-29 accumulation appears wild-type in animals in which the array used for mosaic analysis has been integrated into the genome (see Materials and Methods). LIN-29 does not accumulate in the B cell lineage until the third larval stage, approximately 38 h and five cell divisions (in the B.a lineage) after the birth of the B cell and approximately 44 h and 11 cell divisions after the birth of AB.prp. It is also formally possible that *lin-29* mRNA could be transcribed prior to array loss but translated later in the lineage. However, it is unlikely that sufficient *lin-29* mRNA transcribed prior to an AB.prp loss would survive and be partitioned to the B cell lineage to provide *lin-29* function during the L3 and later stages. Instead, we favor a model of partial redundancy in spicule length determination; there is a major requirement for *lin-29(+)* in the B cell lineage, but another cell lineage (or lineages) contributes to spicule length in a *lin-29*-dependent fashion and can sometimes compensate for loss of *lin-29(+)* in the B lineage. The identity of the additional cell(s) is not known. Assuming that any additional AB.pr losses will have short spicules (Fig. 6A), the two AB.prp losses with wild-type-length spicules suggest that the additional cell(s) providing *lin-29(+)* activity descend from AB.pra. AB.pra gives rise to the male-specific blast cells V5.r and T.r and in approximately half of males to P11. LIN-29-accumulating cells that are derived from these lineages include hypodermal cells and cells of the preanal ganglia. P11 can arise from AB.pra, a lineage largely symmetrical to AB.plp and which also gives rise to V5.l and T.l. No losses within these lineages produced short spicules, indicating that *lin-29* loss in either one of these lineages is insufficient to produce a mutant phenotype. In fact, no short spicules were observed among 32 *lin-29*-mosaics with losses dispersed throughout the lineage but not involving B progeny cells (Fig. 6A). This pattern of mosaics indicates that *lin-29(+)* in the B cell lineage is sufficient for wild-type-length spicule formation.

Many other genes have been identified that function in spicule development. For example, mutations in members of the *daf-4/sma-2* TGF-β-like pathway lead to males with mutant tails including crumpled spicules (Savage *et al.*, 1996), the *lin-44/lin-17* Wnt signaling system is required to generate the proper asymmetry within the B cell lineage (Sternberg and Horvitz, 1988; Herman and Horvitz, 1994; Herman *et al.*, 1995; Sawa *et al.*, 1996), and the *lin-3/let-23* pathway mediates inductive signaling that instructs B cell lineage fates (Chamberlin and Sternberg, 1994). Each of these signaling systems is required for proper spicule formation, and the latter two are known to specify the fate of the B cell and its progeny.

Since the B cell lineage appears to be normal in *lin-29* mutant males, it is unlikely that these B cell fate specification signals are perturbed. Instead, the defect in *lin-29* mutants appears to be in a late step of spicule morphogenesis. Such a function is analogous to the role of *lin-29* in vulva formation. Vulval development in *lin-29* mutant hermaphrodites is normal until exit from the fourth molt at which time a morphogenesis defect is observed (Bettinger *et al.*, 1997). However, in contrast to the major cell autonomous requirement for *lin-29(+)* in the B cell lineage,

lin-29(+) was found to be required in the lateral hypodermis overlying the vulva, thereby playing an ancillary and structural role in vulva morphogenesis, rather than a direct role within the vulval cells (Bettinger *et al.,* 1997).

The function of *lin-29* in the morphogenesis of spicules is currently unknown. Because *lin-29* is known to regulate collagen gene expression in the hypodermis (Liu *et al.,* 1995; Rougvie and Ambros, 1995), one role for *lin-29* in the male tail may be to regulate the expression of certain collagens, such as those that provide the cuticle covering of the spicules. Accumulation of an abnormal complement of collagen proteins could result in spicules of altered morphology. Consistent with this hypothesis, LIN-29 is detected in the spicule socket cells, cells which probably secrete the spicule cuticle (White, 1988; L. Jiang and P. Sternberg, personal communication) and *lin-29* mutant spicules appear to lack the rigidity of wild-type spicules (see Table 2 legend). LIN-29 also accumulates in B.paa, a cell that secretes cuticle of the gubernaculum (Sulston *et al.,* 1980).

Mutations in *lin-4, lin-14,* and *lin-28,* upstream temporal regulators of *lin-29,* cause spicule defects that are more severe than those observed in *lin-29* mutants. Males bearing mutations in these genes produce little (*lin-14* and *lin-28* mutants) or no (*lin-4* mutants) detectable spicule material (Fig. 2). Cell lineage analysis of *lin-14* mutant males reveals precocious divisions in the F, U, and Y lineages (Ambros and Horvitz, 1984). Because progeny of these cells provide inductive signals to the B cell (Chamberlin and Sternberg, 1993, 1994), it is likely that B cell specification is also abnormal in *lin-14* mutants. The B cell division patterns have not been reported for *lin-14* or *lin-28* mutants. In *lin-4* mutant males, the lineages of all four cells, B, F, U, and Y, are abnormal (Chalfie *et al.,* 1981) and no spicule material is observed. The absence of identifiable spicule material in *lin-4* mutants suggests that the B cell progeny fates are not specified.

The male tail defects observed in *lin-4, lin-14,* and *lin-28* mutants are more pleiotropic than those of *lin-29* mutants. *lin-4* mutations cause developmental timing alterations that can generally be described as the reiteration of L1 stage-specific cell division patterns during subsequent stages in a diverse array of cell types (Ambros and Horvitz, 1984). In the male tail, reiteration of L1 stage-specific division patterns in the V5, V6, and T cell lineages prevents execution of the ray sublineages and, as a consequence, rays are not formed. Loss of *lin-28* function causes temporal alterations of cell fate that are interpreted as deletion of L2 stage-specific cell division patterns. Deletion of these L2 stage-specific division patterns in the V5, V6, and T lineages results in precocious execution of the ray sublineages. The rays are reduced in number because proliferative divisions have been deleted (Ambros and Horvitz, 1984). In contrast to the many defects observed in these upstream heterochronic mutants, the only cell division defect observed in lineages that contribute to the male tail in *lin-29* mutants is the reiterative division of the lateral hypodermal seam cell produced by V5.l and V5.r (Ambros and Horvitz, 1984).

lin-29 is classified as a member of the heterochronic gene pathway because its mutation results in reiteration of cell division and larval cuticle synthesis in the lateral seam, an indefinite delay in the timing of seam cell terminal differentiation. In another sense, LIN-29 may be defined as a terminal differentiation factor, because *lin-29* is not necessarily required for the timing of seam cell terminal differentiation; rather, it may be viewed as an effector of the timing signal conveyed by *lin-4, lin-14,* and *lin-28.* Interpretation of the male tail phenotype of *lin-29* mutants is more consistent with this latter view of *lin-29* function. The male tail defects are not heterochronic per se, but rather involve a failure to execute properly a stage-specific morphogenetic event. Consistent with the role of *lin-29* in other developmental events, *lin-29* function in spicule morphogenesis is also restricted to the L4 molt and adult stage. With the exception of their spicules, *lin-29* mutant male tails appear relatively normal. Thus, *lin-29* is required for terminal differentiation of the lateral seam and morphogenesis of the hermaphrodite vulva and male tail but is not required for generation of all adult-specific structures.

Expression and Roles of LIN-29 in Other Male-Specific Cells

Although mosaic animals lacking *lin-29(+)* in the P1 lineage have wild-type-length spicules, 10 of 11 of these animals failed to produce cross-progeny in mating tests. This result suggests an additional male-mating requirement for *lin-29(+)* in the P1 lineage. An additional defect was observed when these P1(−) mosaic animals were anesthetized for Nomarski microscopy: their spicules failed to protract from the gubernaculum. This defect is not due to blockage of the cloacal opening as the mosaic animals were not constipated and their spicule tips protruded briefly as the animals became anesthetized. This requirement for *lin-29(+)* traces, at least in part, to the P2 lineage that produces mainly body wall muscle and hypodermal cells.

In addition to the B cell progeny, LIN-29-accumulating male tail cells include the male SET, many neurons of the ventral cord, and the LC (Fig. 3). In *lin-29* mutant males the lateral hypodermal seam remains undifferentiated, as shown by the failure of these cells to fuse (Ambros and Horvitz, 1984). In contrast, the SET fuses in *lin-29* mutants, as it does in wild-type males. While *lin-29* is not required for SET fusion, LIN-29 does accumulate in the SET nuclei at the same time as in the nuclei of the body seam. The role of LIN-29 in these cells is probably not related to spicule length, as elimination of the SET by laser microsurgery does not affect spicule length and only causes a slight attenuation of the fan (Sulston *et al.,* 1980). In the absence of LIN-29, the male SET may continue to express larval collagen genes following the fourth molt, but since the SET does not produce alae, there is not an obvious morphological result of this defect.

The decreased frequency of behaviors involving backward movement during mating in *lin-29* mutants (Table 2) may reflect a function for LIN-29 in ventral cord neurons. Consistent with a male-specific role for LIN-29 in these cells, LIN-29 is not detectable in the ventral cord of hermaphrodites at any stage. Among the LIN-29-accumulating cells in the ventral cord are P11.a progeny cells and *unc-4::lacZ*-expressing cells that are likely to be VA and DA motor neurons, neurons known to be involved in backward movement.

LIN-29 accumulation in the linker cell probably reflects a partial *lin-29*-dependent function in male gonad migration. In hermaphrodites, LIN-29 accumulates in the distal tip cells (DTC) (Bettinger *et al.*, 1996), cells that perform at least two discrete functions: they lead the migration of the gonad arms, and they maintain the germline stem cell population at the distal end of each arm. In males, these two functions are carried out by two different cell types. The LC leads the migration of the developing gonad from the midbody region to the cloaca and the two DTCs, which remain positioned at the distal end of the gonad, maintain the germline population (Kimble, 1981). In wild-type males, LIN-29 accumulates in the LC beginning during the L3 stage and it remains detectable until the LC is induced to die as the gonad fuses to the cloaca. LIN-29 is not detected in the male DTCs at any stage. Thus, the accumulation pattern of LIN-29 suggests a role for LIN-29 in gonad migration. Consistent with this possibility, *lin-29(0)* males have a gonad migration defect, albeit at low to moderate penetrance (Table 3). The defects observed include faulty posterior and dorsal turns and delays in the timing of posterior migration. The partial penetrance of the gonad phenotypes suggests that *lin-29* functions redundantly in this process. Genes controlled by the LIN-29 transcription factor in the LC may encode products involved in the reception of, or response to, gonadal migration guidance cues.

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